

# Vegetative growth, compatible solute accumulation, ion partitioning and chlorophyll fluorescence of ‘Malas-e-Saveh’ and ‘Shishe-Kab’ pomegranates in response to salinity stress

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## Abstract

The present research was conducted to assess physiological responses of ‘Malas-e-Saveh’ (Malas) and ‘Shishe-Kab’ (Shishe) pomegranates to water of different salt content and electrical conductivity (1.05, 4.61, and 7.46 dS m<sup>-1</sup>). Both cultivars showed a reduced trunk length due to salinity. Relative water content and stomatal conductivity of both cultivars were significantly reduced under salt stress, but ion leakage increased. In both cultivars, total chlorophyll (Chl) and carbohydrates decreased with rise in salinity, while proline accumulation increased. With salinity increment, the Chl fluorescence parameters (maximum photochemical efficiency of PSII and effective quantum yield of PSII) declined significantly in both cultivars, with higher reduction observed in Shishe. Generally, more Na<sup>+</sup> accumulated in shoots and more Cl<sup>-</sup> was observed in leaves. Cl<sup>-</sup> accumulation increased by salinity in leaves of Malas, but it was reduced in Shishe. The K<sup>+</sup>/Na<sup>+</sup> ratio in leaves decreased in both cultivars by salinity increment. Malas was less affected by osmotic effects of NaCl, but it accumulated more Cl<sup>-</sup> in its leaves. Thus, Malas might be more affected by negative effects of salinity.

*Additional key words:* chloride; fluorescence; growth; NaCl; potassium; proline; sodium; total carbohydrates.

## Introduction

Pomegranate (*Punica granatum* L., Punicaceae) is an important horticultural crop for both domestic and export markets in Iran. It has been cultivated widely in arid and semiarid regions that face salinity stress (Ranjbar 1981, Khoshgoftarmanesh and Siadat 2002). Nowadays, commercial orchards of pomegranate trees can be seen in the Mediterranean basin and Asia (Holland *et al.* 2009). Of the current 230 million ha of irrigated land, 45 million ha (20%) are affected (FAO 2008) by primary and/or secondary salinity (Chapman 1966). Primary salinity arises from weathered rocks, capillary rise from shallow brackish groundwater, salt laden sand blown by sea winds, and impeded drainage. Secondary salinization is the result of human activities, such as fields irrigated without proper

drainage system, industrial effluents, excessive lands with natural plant cover removed, flooding with salt-rich waters, high water table, and irrigation with poor-quality groundwater (Mane *et al.* 2011). Under salinity stress, plant growth and development is affected negatively by water stress (*i.e.*, by lowering osmotic potential of soil solution and thus reducing water uptake) or by ionic stress (*i.e.*, by nutritional imbalance and/or toxicity), or by the combination of the mentioned factors (Ashraf 1994, Marschner 1995, Ashraf and Harris 2004, Silva-Ortega *et al.* 2008). Water stress results from high solute concentration, while ionic stress relates to altered Na<sup>+</sup>/K<sup>+</sup> and Na<sup>+</sup>/Ca<sup>2+</sup> ratios or high, harmful Na<sup>+</sup> and Cl<sup>-</sup> concentrations (Apse and Blumwald 2007). Nutrient

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**Abbreviations:** ABA – abscisic acid; C – control; Chl – chlorophyll; DM – dry mass; EC – electrical conductivity; F<sub>0</sub> – the minimal fluorescence in the dark-adapted state; Fe – iron; F<sub>m</sub> – the maximal fluorescence in the dark-adapted state; FM – fresh mass; F<sub>s</sub> – minimum Chl fluorescence in the light-adapted state; F<sub>v</sub> – the variable fluorescence; F<sub>v</sub>/F<sub>m</sub> – maximum PSII photochemical efficiency; F<sub>m</sub>' – maximum Chl fluorescence in the light-adapted state; g<sub>s</sub> – stomatal conductance; LIL – leaf ion leakage; Malas – Malas-e-Saveh; q<sub>N</sub> – nonphotochemical quenching; q<sub>P</sub> – photochemical quenching; RWC – relative water content; S1 – EC of 4.61 dS m<sup>-1</sup>; S2 – EC of 7.46 dS m<sup>-1</sup>; Shishe – Shishe-Kab; SLA – specific leaf area; SLM – specific leaf mass; SSP – soil-saturated paste; TD – trunk diameter; TL – trunk length; TM – turgid mass; Φ<sub>PSII</sub> – effective quantum yield of photochemical energy conversion in PSII.

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uptake can be affected by  $\text{Na}^+$  and  $\text{Cl}^-$  ions through competition or changing membrane selectivity, *e.g.*, high concentration of  $\text{Na}^+$  increases potassium and/or calcium deficiency (Tester and Davenport 2003). Munns and Tester (2008) defined salinity resistance as the ability to maintain adequate growth and metabolism under stress. Paranychianakis and Angelakis (2008) reported that distinction between salt-tolerant and -sensitive genotypes is associated with mechanism of ion uptake and accumulation patterns into different organs. Smekens and Vantinderen (2001) showed that salt treatments result in thicker leaves and higher leaf dry matter. In glycophytes, such as citrus and rose, NaCl stress symptoms are observed in lower leaves and they are distributed to upper ones after salinity increment (Banuls and Primo-Millo 1995, Weber and Reimann-Phillip 1989). Wahome (2003) reported that plants may lose some sections of their shoots under such conditions. Bongi and Loreto (1989) showed that photosynthesis is reduced partly because of the reduced mesophyll conductance caused by leaf thickening in salt-stressed olive leaves. The cortex/stele ratio in olive plants increased under salinity because of water deficit (Karimi *et al.* 2009). Marschner (1995) stated that plant species differ greatly in their growth response to salinity, thus they can be categorized into two groups: (1) halophytes grow optimally at relatively high NaCl concentration (400 mM), and (2) glycophytes show relatively low salt tolerance and their growth is severely inhibited even at low salinity levels. Plants adopt 3 physiological strategies to cope with excessive amounts of  $\text{Na}^+$  and  $\text{Cl}^-$  in the root medium: (a) osmotic stress tolerance, (b)  $\text{Na}^+$  and  $\text{Cl}^-$  exclusion from leaves, and (c) tissue tolerance to  $\text{Na}^+$  and  $\text{Cl}^-$  accumulation (Zhu 2003, Eitan *et al.* 2005, Munns *et al.* 2006, Ashraf and Foolad 2007, Molinari *et al.* 2007, Martinez-Rodriguez *et al.* 2008, Munns and Tester 2008). Thus, plants respond to salinity at nonstomatal and/or stomatal levels. Nonstomatal responses include instability of pigment-protein complexes and destruction of chloroplast structure (Zaman *et al.* 2002), which inhibits photosynthetic activities (Matos *et al.* 2004, Rouhi *et al.* 2006). At the stomatal level, the stomatal activity is

reduced and it limits photosynthesis (Lawlor and Cornic 2002) and photon flux energy used for photochemistry (Cornic 1994). Chlorophyll (Chl) fluorescence yield can show stress or damage to the photosynthetic apparatus (Glynn *et al.* 2003). Various reports focus on Chl fluorescence parameters in plants subjected to drought (Petsas and Grammatikopoulos 2009, Khaleghi *et al.* 2012), freezing (Percival and Fraser 2001), ozone (Meinander *et al.* 1996), and salinity (Ranjbarfordoei *et al.* 2006, García-Sánchez and Syvertsen 2006, Kalaji *et al.* 2011). Jain and Dass (1988) and Patil and Waghmare (1982) reported that plant height, number of leaves, and stem diameter of pomegranate plants decreased significantly with increasing soil salinity. They recommended that pomegranate should not grow in soils with electrical conductivity (EC) of saturation paste more than  $10 \text{ dS m}^{-1}$ . Doring and Ludders (1986, 1987) and Naeini *et al.* (2006) reported that salinity strongly reduced growth parameters of pomegranates and found the highest accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in roots and leaves, respectively. Bhandana and Lazarovitch (2010) listed pomegranate as moderately sensitive. Holland *et al.* (2009) noted that pomegranates are amenable to irrigation with saline water (EC of 2.5 to  $4.0 \text{ dS m}^{-1}$ ) and they produce a normal yield. Levin (2006) reported positive response to irrigation with recycled water in pomegranate orchards in Turkmenistan. Malas and Shishe are commercial Iranian cultivars used for export markets. Both cultivars are late-ripening, medium to large size, with red skin and arils (Varasteh *et al.* 2006), but different in their shape. Although there are some reports about pomegranate responses to NaCl, no study refers to salinity responses of these cultivars under field conditions. Moreover, Chl fluorescence has not been studied in pomegranates under salt stress. Therefore we studied the physiological mechanisms operating at the whole-plant and cellular levels in these pomegranate cultivars under NaCl stress. We aimed also to estimate their abilities to exclude  $\text{Na}^+$  or  $\text{Cl}^-$ . Our results could be used in a breeding program designed to enhance salt tolerance in Iranian pomegranates.

## Materials and methods

**Plants, growth conditions, and treatments:** The research was carried out using one-year-old, bare rooted pomegranate plants of Malas and Shishe cultivars under the field conditions at Salinity Research Station of University of Birjand ( $32^\circ 52' \text{N}$  and  $52^\circ 12' \text{E}$ ). During 2011 and 2012, annual precipitation was *ca.* 171 mm, the lowest and the highest temperatures were  $-5$  and  $+40^\circ \text{C}$ , respectively, based on 50-year data of South Khorassan, Iran. A cultivar selection was done according to fruit yields and their quality (Varasteh *et al.* 2006). Plants grew in a loamy soil with the space among plants of  $2 \times 4 \text{ m}$  in rectangular pattern (*see* the text table below).

As salinity treatments, plants were irrigated by water of different EC: C (control), S1 ( $\text{EC } 4.61 \text{ dS m}^{-1}$ ), and S2 ( $\text{EC } 7.46 \text{ dS m}^{-1}$ ) (Table 1). Irrigation was done according to calibration by data collected from evaporation pan (class A), oven method (Black 1965), pressure plates (Roades 1982), and tensiometer (Santa Barbara, USA) reading [ $0.03 \text{ MPa}$ ,  $30 \text{ KPa}$  or field capacity] to avoid water stress in plants. For equal irrigation, the volume of water was calculated using counters connected to each pipe. At the end of experiment, saturated pastes of treated soils were analyzed (Table 2).

|                               |                          |
|-------------------------------|--------------------------|
| EC                            | 0.93 dS m <sup>-1</sup>  |
| pH                            | 7.12                     |
| Total N                       | 0.08%                    |
| P                             | 8 mg kg <sup>-1</sup>    |
| K <sup>+</sup>                | 210 mg kg <sup>-1</sup>  |
| Zn <sup>2+</sup>              | 0.63 mg kg <sup>-1</sup> |
| Cu <sup>2+</sup>              | 0.25 mg kg <sup>-1</sup> |
| Mn <sup>2+</sup>              | 1.96 mg kg <sup>-1</sup> |
| Fe <sup>2+</sup>              | 2.65 mg kg <sup>-1</sup> |
| Na <sup>+</sup>               | 4 meq l <sup>-1</sup>    |
| Mg <sup>2+</sup>              | 3.14 meq l <sup>-1</sup> |
| Ca <sup>2+</sup>              | 2.6 meq l <sup>-1</sup>  |
| Cl <sup>-</sup>               | 0.5 meq l <sup>-1</sup>  |
| HCO <sub>3</sub> <sup>-</sup> | 0.3 meq l <sup>-1</sup>  |

**Growth parameters:** On each plant, the trunk length (TL) and trunk diameter (TD) were assessed during the growing season. TL was measured *via* assessment of vertical line related to the soil surface, from the highest to the lowest point of the tree. For TD, the basal part of the mentioned shoot was evaluated by Vernier calliper.

Leaf discs (0.5 cm<sup>2</sup>) were cut and oven-dried under 100°C for 48 h. Then specific leaf mass (SLM) and specific leaf area (SLA) were calculated using method of Hunt (1990):

$$SLM = \frac{\text{Disc dry mass}}{0.5} \quad SLA = \frac{0.5}{\text{Disc dry mass}}$$

**Leaf ion leakage (LIL):** Large leaf segments were cut out at random, washed 3 times with distilled water in order to remove surface contaminants, and then placed individually in stoppered vials containing 10 ml of distilled water. Consequently, they were incubated at room temperature (25°C) on a shaker (100 × g) for 24 h to measure EC of the solution (EC<sub>1</sub>). Then the same vials with leaf samples were placed in an autoclave at 120°C for 20 min and the 2<sup>nd</sup> measurement of conductivity (EC<sub>2</sub>) was done after cooling the solution to room temperature. The ion leakage was calculated as  $\frac{EC_1}{EC_2}$  (Lutts *et al.* 1995).

**Relative water content (RWC):** Leaf discs (1.5 cm<sup>2</sup>) were weighed to determine the fresh mass (FM), soaked in distilled water at 25°C for 4 h to determine the turgid mass (TM), then oven-dried at 80°C for 24 h to determine the dry mass (DM). Finally, following equation was used to calculate RWC (Barrs and Weatherley 1962):

$$RWC [\%] = \frac{(FM-DM)}{(TM-DM)} \times 100$$

**Stomatal conductance (g<sub>s</sub>)** was determined by leaf porometer (DECAGON DEVICES, INC., Pullman, USA) during the growing season. Measurements were done at 25°C at 9:00 to 12:00 h.

**Chl, proline, and total soluble carbohydrates:** Chl content was determined by method of Saini *et al.* (2001) using 80% acetone or by SPAD 502 meter (MINOLTA, Osaka, Japan). Leaf discs of 0.25 g (FM) were extracted in 5 ml of acetone (80%), then centrifuged for 10 min in 8,000 × g. The supernatant was used to make a final volume of 50 ml of the leaf extract. Extraction of leaf tissue with the buffer continued until decoloration. Absorbance of the extract was read at 645 and 663 nm with a spectrophotometer (SHIMADZU AA-670, Japan) and 80% acetone was used as a blank. Finally, total leaf Chl content was calculated according to the following equation:

$$\text{Total Chl} = \frac{(20.2 A_{645} + 8.02 A_{663}) \times V}{W} \times 1000,$$

where V is volume (50 ml), W is fresh mass of the leaf disc (0.25 g), A<sub>645</sub> and A<sub>663</sub> are absorbances at 645 and 663 nm, respectively.

Proline was determined by the ninhydrin method described by Bates *et al.* (1973), using L-proline as a standard (0–500 μmol, MERCK). Leaf samples of 0.5 g (FM) were homogenized in 10 ml of 3% (w/v) aqueous sulfo-salicylic acid and centrifuged for 30 min at 14,000 × g.

Table 1. Evaluation of water quality used in the experiment. EC = 1.05 dS m<sup>-1</sup> was used as control (C). S1 – EC of 4.61 dS m<sup>-1</sup>; S2 – EC of 7.46 dS m<sup>-1</sup>.

| Treatment | EC of H <sub>2</sub> O [dS m <sup>-1</sup> ] | pH   | Ca <sup>2+</sup> [meq l <sup>-1</sup> ] | Mg <sup>2+</sup> [meq l <sup>-1</sup> ] | K <sup>+</sup> [meq l <sup>-1</sup> ] | Na <sup>+</sup> [meq l <sup>-1</sup> ] | Cl <sup>-</sup> [meq l <sup>-1</sup> ] | HCO <sub>3</sub> <sup>-</sup> [meq l <sup>-1</sup> ] |
|-----------|--|------|---|---|---------------------------------------|--|--|--|
| 1         | 1.05 (C)                                     | 7.54 | 2.1                                     | 5.8                                     | 0.2                                   | 8.5                                    | 13.0                                   | 3.9  |
| 2         | 4.61 (S1)                                    | 7.41 | 7.0                                     | 18.4                                    | 0.7                                   | 23.8                                   | 14.0                                   | 2.3  |
| 3         | 7.46 (S2)                                    | 7.26 | 12.7                                    | 25.0                                    | 1.1                                   | 77.3                                   | 63.0                                   | 0.9  |

Table 2. Evaluation of soil saturated paste in each treatment. Treatment 1 was used as control.

| Treatment | EC of H <sub>2</sub> O [dS m <sup>-1</sup> ] | pH   | Na <sup>+</sup> [meq l <sup>-1</sup> ] | Cl <sup>-</sup> [meq l <sup>-1</sup> ] | K <sup>+</sup> [meq l <sup>-1</sup> ] |
|-----------|--|------|--|--|---------------------------------------|
| 1         | 2.35   | 8.20 | 15.11                                  | 1.52                                   | 4.45                                  |
| 2         | 7.13   | 8.05 | 67.73                                  | 1.75                                   | 4.45                                  |
| 3         | 9.26   | 8.04 | 86.29                                  | 2.01                                   | 4.19                                  |

Ninhydrin (2 ml) and glacial acetic acid (2 ml) were added to the supernatant and the mixture was boiled at 100°C for 1 h and then placed in an ice bath to stop the reaction. After extraction with toluene, free proline was quantified at 520 nm using a spectrophotometer (SHIMADZU AA-670, Japan). Equation used for standard curve preparation was  $y = 252.38x - 8.25$  ( $R^2 = 0.90$ ).

The total leaf soluble carbohydrates were determined according to Irigoyen *et al.* (1992) and glucose (0–100 mg l<sup>-1</sup>, from MERCK) was used as a standard. Leaf samples of 0.5 g(FM) were homogenized in 5 ml ethanol (95%) and centrifuged at  $4,500 \times g$  for 15 min, the supernatant was removed from the sample and the residue was resuspended in 5 ml of 70% ethanol. Then the supernatant was centrifuged again for final extraction. Both supernatants were combined. Anthrone-sulfuric acid assay was used for determination. An aliquot of 100 µl was added to 3 ml of anthrone-sulfuric acid solution and the mixture was shaken, heated in a boiling water bath for 10 min and cooled at 4°C. The absorption at 625 nm was determined by spectrophotometer (SHIMADZU AA-670, Japan). Equation used for standard curve preparation was  $y = 545.04x - 29.973$  ( $R^2 = 0.94$ ).

**Chl fluorescence** was measured on the top, attached, and dark-adapted leaf of each plant using a MINI PAM fluorometer (WALZ, Effeltrich, Germany) according to the protocol of Genty *et al.* (1989). Leaves were kept for 30 min in the dark-adapted state using light-exclusion clips. At this state, all reaction centers and electron carriers of PSII are reoxidized, which is necessary for the rapid induction of fluorescence. Under such condition, non-photochemical quenching ( $q_N$ ) is relaxed to its minimum value (Roháček 2002, Zhang and Xu 2003). Low-intensity modulated light ( $<0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was used to measure

the minimum fluorescence ( $F_0$ ). The maximum fluorescence ( $F_m$ ) was obtained by 0.3-s pulses of saturating light of 20,000 Hz. The maximum photochemical efficiency of PSII,  $F_v/F_m$ , was calculated according to Kitajima and Butler (1975). Concurrently, both the minimum Chl fluorescence in the light-adapted state ( $F_s$ ) and the maximum Chl fluorescence in the light-adapted state ( $F_m'$ ) were measured. The effective quantum yield of photochemical energy conversion in PSII was calculated according to Genty *et al.* (1989) as:  $\Phi_{\text{PSII}} = \frac{(F_m' - F_s)}{F_m'}$

**Nutrient analysis and current shoot growth:** Chemical analysis was carried out with oven-dried samples of leaves and current shoot tissues, which were ground separately and ashed at 550°C for 90 min in a porcelain crucible. Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> were determined in these samples. The ash was resuspended in hot 2 M HCl, filtered, made up to 50 ml with distilled water, and then used for Na<sup>+</sup> and K<sup>+</sup> analysis with a flame photometer (CORNING 405, Cambridge, UK). Concentration of Cl<sup>-</sup> was assessed by the titration method (Chapman and Pratt 1982).

**Statistical analysis:** The experiment was set up in a completely randomized block design, consisting of three blocks, each block consisted of three saline treatments and two cultivars (each cultivar containing 30 trees), where salinity was used as a main factor. We assumed that all the measured data came from normal (Gaussian) data distribution even if it was not always true, especially, in the case of fluorescence measurements (Lazár *et al.* 1998, 2006). Statistical analysis of data was carried out using analysis of variance (ANOVA) procedure on GENSTAT. The averages were compared with Duncan's multiple range test at 5% level.

## Results

**Water and soil analysis:** The final EC of soil-saturated paste (SSP) increased to 2.35, 7.13, and 9.26 dS m<sup>-1</sup> in C, S1, and S2 treatments, respectively. The Na<sup>+</sup> and Cl<sup>-</sup> concentrations in SSP rose with increasing content of these ions in the irrigation water (Table 1). Although K<sup>+</sup> concentration of irrigation water increased with salinity increment, the lowest K<sup>+</sup> in SSP was found in EC = 7.46 dS m<sup>-1</sup> (Table 2).

**Growth parameters, LIL, and RWC:** The highest TL was found in C treatment for both cultivars. The significant difference in TL was observed among S2 and S1 treatments. Although TL was reduced in S1, it increased at S2 (Table 3). TD of both cultivars affected significantly salinity, compared with C. In Malas, TD increased significantly with salinity, but it decreased in Shishe cultivar. SLM was unaffected by different treatments, however, SLA showed significant change. The highest SLA was at S1 in both cultivars and it declined significantly with

further salinity increment. Moreover, the lowest value was observed in Shishe (Table 3). LIL of Malas remained unaffected at different levels of salinity, however, increasing salt stress enhanced significantly LIL in Shishe. RWC of both cultivars declined by salinity increment, the lowest values were found at S1. Moreover, the lowest RWC were shown in Shishe under both S1 and S2 treatments (Table 3).

**$g_s$ , Chl content, proline, and total soluble carbohydrates:** Malas showed significant reduction in  $g_s$  under salinity, compared with C. In Shishe,  $g_s$  increased significantly from C to S1 treatments, and then declined in S2 (Table 4).

Total Chl content in both cultivars lowered significantly with increasing salinity; the lowest amount was observed in S1. In both cultivars, higher Chl content was found in S2 compared with S1 treatment (Table 4). Proline accumulation in Malas declined significantly from C to S1

Table 3. Effects of salinized waters on trunk length (TL) and diameter (TD), specific leaf mass (SLM), specific leaf area (SLA), leaf ion leakage (LIL), and relative water content (RWC). EC = 1.05 d S m<sup>-1</sup> was used as control. Mean values in each column followed by *the same letter* are not significantly different ( $P < 0.05$ ) by the *Duncan's* multiple range test.

| EC of H <sub>2</sub> O [dS m <sup>-1</sup> ] | TL [cm]            | TD [mm]            | SLM [g cm <sup>-2</sup> ] | SLA [cm <sup>2</sup> g <sup>-1</sup> ] | LIL [%]              | RWC [%]            |
|--|--------------------|--------------------|---------------------------|--|----------------------|--------------------|
| <b>Malas-e-Saveh</b>                         |                    |                    |                           |  |                      |                    |
| 1.05 (C)                                     | 78.10 <sup>b</sup> | 10.12 <sup>c</sup> | 0.011 <sup>a</sup>        | 95.01 <sup>c</sup>                     | 0.153 <sup>bc</sup>  | 71.17 <sup>b</sup> |
| 4.61 (S1)                                    | 63.00 <sup>e</sup> | 10.40 <sup>b</sup> | 0.010 <sup>a</sup>        | 104.27 <sup>b</sup>                    | 0.193 <sup>ab</sup>  | 69.00 <sup>d</sup> |
| 7.46 (S2)                                    | 70.30 <sup>d</sup> | 10.65 <sup>a</sup> | 0.010 <sup>a</sup>        | 98.97 <sup>c</sup>                     | 0.187 <sup>abc</sup> | 70.67 <sup>c</sup> |
| <b>Shishe-Kab</b>                            |                    |                    |                           |  |                      |                    |
| 1.05 (C)                                     | 87.60 <sup>a</sup> | 10.38 <sup>b</sup> | 0.011 <sup>a</sup>        | 97.69 <sup>d</sup>                     | 0.130 <sup>c</sup>   | 71.83 <sup>a</sup> |
| 4.61 (S1)                                    | 58.60 <sup>f</sup> | 8.97 <sup>e</sup>  | 0.010 <sup>a</sup>        | 113.57 <sup>a</sup>                    | 0.163 <sup>bc</sup>  | 66.17 <sup>f</sup> |
| 7.46 (S2)                                    | 73.00 <sup>c</sup> | 9.43 <sup>d</sup>  | 0.010 <sup>a</sup>        | 84.30 <sup>f</sup>                     | 0.243 <sup>a</sup>   | 67.67 <sup>e</sup> |

Table 4. Effects of salinized water on leaf total chlorophyll (Chl), stomatal conductance ( $g_s$ ), proline, and total carbohydrate contents. EC = 1.05 dS m<sup>-1</sup> was used as control. Mean values in each column followed by *the same letter* are not significantly different ( $P < 0.05$ ) by the *Duncan's* multiple range test.

| EC of H <sub>2</sub> O [dS m <sup>-1</sup> ] | Chl [mg g <sup>-1</sup> (FM)] | $g_s$ [mmol m <sup>-2</sup> s <sup>-1</sup> ] | Proline [μmol g <sup>-1</sup> (FM)] | Carbohydrates [mg g <sup>-1</sup> (FM)] |
|--|-------------------------------|---|-------------------------------------|---|
| <b>Malas-e-Saveh</b>                         |                               |   |                                     |   |
| 1.05 (C)                                     | 1.896 <sup>b</sup>            | 151.20 <sup>a</sup>                           | 605.00 <sup>c</sup>                 | 306.30 <sup>c</sup>                     |
| 4.61 (S1)                                    | 1.535 <sup>d</sup>            | 145.70 <sup>c</sup>                           | 577.90 <sup>d</sup>                 | 315.00 <sup>a</sup>                     |
| 7.46 (S2)                                    | 1.612 <sup>c</sup>            | 147.90 <sup>b</sup>                           | 636.00 <sup>a</sup>                 | 292.30 <sup>e</sup>                     |
| <b>Shishe-Kab</b>                            |                               |   |                                     |   |
| 1.05 (C)                                     | 1.991 <sup>a</sup>            | 133.20 <sup>e</sup>                           | 558.80 <sup>f</sup>                 | 315.00 <sup>a</sup>                     |
| 4.61 (S1)                                    | 1.301 <sup>e</sup>            | 133.60 <sup>d</sup>                           | 573.90 <sup>e</sup>                 | 309.70 <sup>b</sup>                     |
| 7.46 (S2)                                    | 1.602 <sup>c</sup>            | 114.10 <sup>f</sup>                           | 605.40 <sup>b</sup>                 | 294.00 <sup>d</sup>                     |

Table 5. Effects of salinized water on current shoot potassium, sodium, and chloride concentrations. EC = 1.05 dS m<sup>-1</sup> was used as control. Mean values in each column followed by *the same letter* are not significantly different ( $P < 0.05$ ) by the *Duncan's* multiple range test.

| EC of H <sub>2</sub> O [dS m <sup>-1</sup> ] | Na <sup>+</sup> [mg g <sup>-1</sup> (DM)] | Cl <sup>-</sup> [mg g <sup>-1</sup> (DM)] | K <sup>+</sup> [mg g <sup>-1</sup> (DM)] | K <sup>+</sup> /Na <sup>+</sup> ratio |
|--|---|---|--|---------------------------------------|
| <b>Malas-e-Saveh</b>                         |   |   |  |                                       |
| 1.05 (C)                                     | 6.81 <sup>d</sup>                         | 0.52 <sup>a</sup>                         | 16.40 <sup>a</sup>                       | 2.42 <sup>a</sup>                     |
| 4.61 (S1)                                    | 8.66 <sup>bc</sup>                        | 0.51 <sup>a</sup>                         | 14.35 <sup>a</sup>                       | 1.73 <sup>c</sup>                     |
| 7.46 (S2)                                    | 11.12 <sup>b</sup>                        | 0.44 <sup>a</sup>                         | 13.62 <sup>a</sup>                       | 1.26 <sup>e</sup>                     |
| <b>Shishe-Kab</b>                            |   |   |  |                                       |
| 1.05 (C)                                     | 6.86 <sup>d</sup>                         | 0.41 <sup>a</sup>                         | 16.00 <sup>a</sup>                       | 2.33 <sup>b</sup>                     |
| 4.61 (S1)                                    | 12.00 <sup>a</sup>                        | 0.55 <sup>a</sup>                         | 13.67 <sup>a</sup>                       | 1.14 <sup>f</sup>                     |
| 7.46 (S2)                                    | 11.12 <sup>b</sup>                        | 0.65 <sup>a</sup>                         | 14.03 <sup>a</sup>                       | 1.42 <sup>d</sup>                     |

and then increased in S2. In Shishe, proline content increased with rising salinity. The highest proline accumulation was obtained at S2 in Malas. Accumulation of total carbohydrates showed an opposite trend compared to proline. In Malas, the highest and lowest carbohydrate contents were observed at S1 and S2, respectively. In Shishe, the carbohydrate content declined with increasing salinity (Table 4).

**Chl fluorescence parameters** were strongly influenced

by salinity stress. In both cultivars,  $F_0$  showed the lowest value under C. It increased significantly with salinity increment (data not shown), while  $F_m$  declined continuously compared with C. The lowest value was observed in Shishe under S2 (data not shown). With increasing salinity, the ratio of  $F_0/F_m$  significantly increased in both cultivars, compared with C, and the highest value was observed in Shishe under S2. No significant difference was found between S1 and S2 treatments in Malas (Fig. 1). With increasing salinity, the  $F_v/F_m$  was significantly

reduced in both cultivars (Fig. 2) and the lowest value was found in Shishe under S2. The ratio of  $F_v/F_m$  decreased significantly as salinity level increased and the lowest ratio was observed in Shishe under S2. In Malas, no significant difference between S1 and S2 treatments (Fig. 3).

The  $\Phi_{PSII}$  (Fig. 4) showed opposite trend and it declined significantly in both cultivars compared with C. The lowest value was observed in Shishe under the highest salinity.

**Nutrient analysis of current shoot and leaf tissues:** As salinity level increased from C to S2,  $Na^+$  accumulated significantly in current shoots of Malas (Table 5). In Shishe,  $Na^+$  content increased from C to S1, while it declined in S2.  $Cl^-$  and  $K^+$  concentrations remained unaffected by cultivar and salinity levels, although  $K^+$  accumulated more than  $Na^+$  (Table 5). In both cultivars,

the  $K^+/Na^+$  ratio decreased significantly with increasing salinity, although the ratio declined from C to S1 and then increased at S2 in Shishe (Table 5). With increasing salinity,  $Na^+$  concentration rose in Malas, however, it showed increment from 1.05 to 4.61 and then reduced from 4.61 to 7.46 dS  $m^{-1}$  in Shishe (Table 6). Significant difference between cultivars appeared in leaf  $Cl^-$  accumulation. With increasing salinity,  $Cl^-$  accumulation in leaves increased significantly in Malas, while it was significantly reduced in leaves of Shishe (Table 6). Accumulation of  $K^+$  increased from C to S1 and then declined in S2 in leaves of Malas, it increased with salinity increment in Shishe.  $K^+$  accumulated more in leaves than  $Na^+$ . The  $K^+/Na^+$  ratio was significantly smaller in leaves of Malas under higher salinity, while it increased from C to S1 and then declined at S2 in Shishe (Table 6).

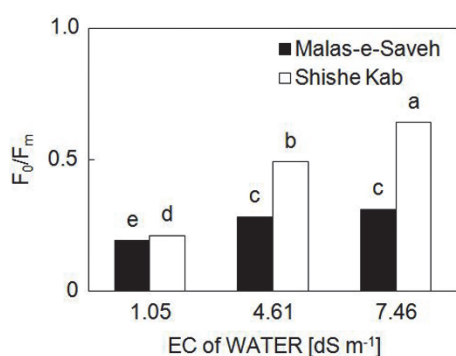


Fig. 1. Evaluation of  $F_v/F_m$  under salinity stress. Means of 30 replications. Bars with the same letters are not significantly different according to Duncan's multiple range test at 5% level.  $F_0$  – the minimal fluorescence in the dark-adapted state,  $F_m$  – the maximal fluorescence in the dark-adapted state.

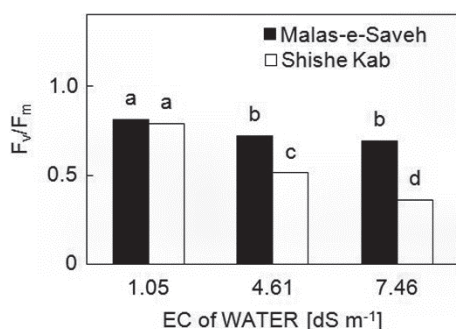


Fig. 2. Evaluation of  $F_v/F_m$  under salinity stress. Means of 30 replications. Bars with the same letters are not significantly different according to Duncan's multiple range test at 5% level.  $F_v$  – variable fluorescence,  $F_m$  – the maximal fluorescence in the dark-adapted state,  $F_v/F_m$  – the maximum quantum yield of PSII or maximum PSII photochemical efficiency.

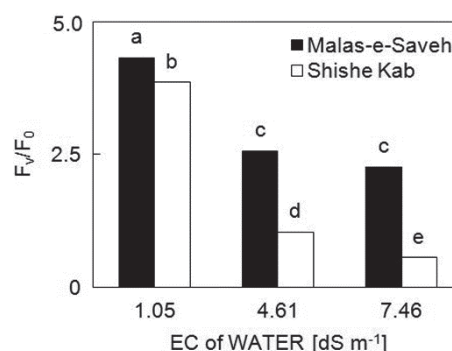


Fig. 3. Evaluation of  $F_v/F_0$  under salinity stress. Means of 30 replications. Bars with the same letters are not significantly different according to Duncan's multiple range test at 5% level.  $F_v$  – variable fluorescence,  $F_0$  – the minimal fluorescence in the dark-adapted state.

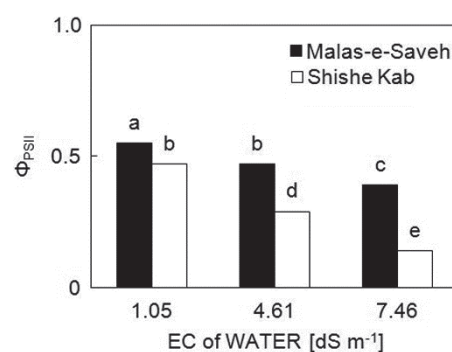


Fig. 4. Evaluation of  $\Phi_{PSII}$  under salinity stress. Means of 30 replications. Bars with the same letters are not significantly different according to Duncan's multiple range test at 5% level.  $\Phi_{PSII}$  – relative quantum yield at steady-state photosynthesis or effective quantum yield of photochemical energy conversion in PSII.

Table 6. Effects of salinized water on leaf potassium, sodium, and chloride concentrations. EC = 1.05 dS m<sup>-1</sup> was used as control. Mean values in each column followed by the same letter are not significantly different ( $P < 0.05$ ) by the Duncan's multiple range test.

| EC of H <sub>2</sub> O<br>[dS m <sup>-1</sup> ] | Na <sup>+</sup><br>[mg g <sup>-1</sup> (DM)] | Cl <sup>-</sup><br>[mg g <sup>-1</sup> (DM)] | K <sup>+</sup><br>[mg g <sup>-1</sup> (DM)] | K <sup>+</sup> /Na <sup>+</sup><br>ratio |
|---|--|--|---|--|
| <b>Malas-e-Saveh</b>                            |  |  |   |  |
| 1.05 (C)  | 4.00 <sup>e</sup>                            | 4.28 <sup>f</sup>                            | 10.61 <sup>f</sup>                          | 2.68 <sup>c</sup>                        |
| 4.61 (S1)                                       | 6.36 <sup>b</sup>                            | 6.82 <sup>c</sup>                            | 16.00 <sup>c</sup>                          | 2.50 <sup>d</sup>                        |
| 7.46 (S2)                                       | 8.01 <sup>a</sup>                            | 8.38 <sup>a</sup>                            | 11.88 <sup>e</sup>                          | 2.13 <sup>e</sup>                        |
| <b>Shishe-Kab</b>                               |  |  |   |  |
| 1.05 (C)  | 3.63 <sup>f</sup>                            | 7.53 <sup>b</sup>                            | 15.94 <sup>d</sup>                          | 4.40 <sup>a</sup>                        |
| 4.61 (S1)                                       | 6.30 <sup>c</sup>                            | 6.72 <sup>d</sup>                            | 16.26 <sup>b</sup>                          | 2.70 <sup>c</sup>                        |
| 7.46 (S2)                                       | 4.18 <sup>d</sup>                            | 5.02 <sup>e</sup>                            | 16.51 <sup>a</sup>                          | 3.96 <sup>b</sup>                        |

**Correlations:** Regression analysis showed a linear correlation ( $R^2 = 0.77$ ) between ion leakage and accumulation of Na<sup>+</sup> in leaves of Malas (data not shown). Ion leakage correlated linearly with K<sup>+</sup> content in leaves of Malas ( $R^2 = 0.54$ ) and Shishe ( $R^2 = 0.91$ ). A negative

correlation was observed between RWC and leaf K<sup>+</sup> content in Malas ( $R^2 = 1$ ) and Shishe ( $R^2 = 0.57$ ). Moreover, proline accumulation in leaves correlated negatively with carbohydrate content in leaves of Malas ( $R^2 = 0.99$ ) and Shishe ( $R^2 = 0.99$ ) (data not shown).

## Discussion

We found that pomegranates could tolerate ECs lesser than 10 dS m<sup>-1</sup> in SSP. It was in agreement with findings of Patil and Waghmare (1982).

The TL affected by salinity stress (Table 3) was reported in pomegranate (Jain and Dass 1988, Patil and Waghmare 1982) and also in olive (Naeini *et al.* 2006, Chen *et al.* 2007, Goreta *et al.* 2007, Ben Ahmed *et al.* 2008). Reduction of TD in Shishe might result from osmotic effects of NaCl on this cultivar (Table 2). Munns and Tester (2008) suggested that moderate salinity inhibits lateral shoot development that becomes apparent over weeks and it is a response to the osmotic effect of NaCl. The highest SLA in both cultivars was found at EC = 4.61 dS m<sup>-1</sup> (Table 3). There was a significant difference among treatments for SLA in Shishe (Table 3), similar with findings of Sergio *et al.* (2012) on chicory, which may result from osmotic effects of salinity on leaf growth of this cultivar. The leaf growth rate decreases when soil salinity increases, primarily due to the osmotic effect of the salt accumulation around the roots (Munns and Tester 2008). This reduction is independent of carbohydrate supply (Munns *et al.* 2000), water status (Munns *et al.* 2000, Fricke and Peters 2002), nutrient deficiency (Hu *et al.* 2005, Hu *et al.* 2007), and ion toxicity (Munns and Tester 2008). Unaffected LIL of Malas might result from high selectivity for K<sup>+</sup> absorption by root and replacement of K<sup>+</sup> by Na<sup>+</sup> in cell membrane structure. On the other hand, with increasing salinity, LIL significantly increased in Shishe (Table 3). It agreed with results in strawberry (Kaya *et al.* 2002a, Khayyat *et al.* 2009a), olive (Goreta *et al.* 2007, Perica *et al.* 2008), and *Echinacea* (Sabra *et al.* 2012). In some species, membrane permeability changed before the growth reduction or before the appearance of severe chlorosis (Lutts *et al.* 1996, Mansour and Salama

2004). RWC decreased first in both cultivars with increasing salinity (S1) and then increased at S2. Both cultivars seemed to improve their water status under salinity (Table 3). It was in agreement with findings in pistachio (Behboudian *et al.* 1986, Hokmabadi *et al.* 2005), but it was contradictory to results from olive (Goreta *et al.* 2007) and rice (Khan and Panda 2008). Some researchers (Torrecillas *et al.* 1995, Hernandez *et al.* 2000) regard osmotic effects as the way how salt stress affects plant water status. Plants may adjust their osmotic status by accumulation of Na<sup>+</sup> and Cl<sup>-</sup> in tissues; however, it may endanger cells and inactivate both photosynthetic and respiratory electron transport (Munns and Tester 2008). Munns and Tester (2008) suggested that salts may build up in the apoplast and dehydrate the cell, which could influence RWC.

Reduction of total Chl contents in both cultivars (Table 4) agreed with results from strawberry (Kaya *et al.* 2002b, Khayyat *et al.* 2009b) and cucumber (Tiwari *et al.* 2010). Although salinity (up to S1) influenced the cultivars via nonstomatal way, Chl increased at S2 (Zaman *et al.* 2002). Salts might build up in the chloroplast and exert a direct toxic effect on photosynthetic processes (Munns and Tester 2008). Sivstev *et al.* (1973) reported that salinity could increase chlorophyllase activity or it may influence absorption of some ions, such as Mg<sup>2+</sup> and Fe<sup>2+</sup>, which are involved in Chl formation (Munns 2002). Grattan and Grieve (1999) reported that reduction of Chl led to the reduction of photosynthetic capacity. The  $g_s$  of Malas was reduced by S1, while it increased at S2. On the other,  $g_s$  decreased with salinity increment in Shishe (Table 4), which was in agreement with raspberry (Neocleous and Vasilakakis 2007) and *Echinacea* (Sabra *et al.* 2012). A reduction of stomata aperture is the most dramatic and

readily measurable parameter of the plant response to salinity. It is undoubtedly induced by the osmotic effect of salinity owing to disturbed water relations and finally local synthesis of abscisic acid (ABA) (Fricke *et al.* 2004). Regulation of  $g_s$  is vital for plants under stress conditions, it prevents from desiccation and improves  $\text{CO}_2$  acquisition (Dodd 2003, Medici *et al.* 2007). Under salinity, leaf turgor and atmospheric vapor pressure are reduced and chemical signals, such as ABA (Kempa *et al.* 2008, Melcher *et al.* 2009), are generated along roots (Chaves *et al.* 2009). Thus, mesophyll conductance is suppressed (Flexas *et al.* 2004, Chaves *et al.* 2009), finally photosynthetic processes might be disrupted and it can affect negatively carbohydrate production. With salinity increment, proline accumulation increased in both cultivars, with higher accumulation in Malas (Table 4). This was in agreement with pistachio (Hokmabadi *et al.* 2005) and cucumber (Tiwari *et al.* 2010). The turgor pressure is controlled by solute regulation within the guard cell protoplast and the RWC of epidermal tissues. Accumulation of inorganic and organic solutes increased the osmotic activity; consequently it reduced water potential and an influx of water from the surrounding cells (Munns and Tester 2008). With salinity increment, accumulation of total carbohydrates was reduced significantly in both cultivars (Table 4), which was in agreement with results of Kaya *et al.* (2002b) for strawberry and of Tiwari *et al.* (2010) for cucumber. Ashraf and Harris (2013) showed that abiotic stress, such as salinity, disrupts carbohydrate production. Salts may build up in the cytoplasm and inhibit enzymes involved in carbohydrate metabolism (Munns and Tester 2008). Reduction of carbohydrate production may be related to (1) the proline accumulation and (2) accumulation and/or disruption of photosynthetic processes involved in carbohydrate production. If  $\text{Na}^+$  and  $\text{Cl}^-$  are sequestered in the vacuole, compatible organic solutes, such as commonly sucrose, proline, and glycine-betaine, must accumulate in the cytosol and in organelles to balance the osmotic pressure of the ions in the vacuole (Flowers *et al.* 1977, Jones *et al.* 1977, Hasegawa *et al.* 2000, Munns 2005). They function as osmolytes and osmoprotectants, stabilizing the tertiary structure of proteins, and serve as an organic nitrogen source (Rhodes *et al.* 2002, Sairam and Tyagi 2004).

Chl fluorescence parameters were strongly influenced by salinity stress. In our experiment,  $F_0$  significantly increased with salinity increment, which was in agreement with Ranjbarfordoei *et al.* (2006) in sweet almond. Rising  $F_0$  occurs when plants are exposed to extreme environmental stress, which leads to the structural alteration in PSII (Krause and Weis 1984).  $F_m$  declined continuously with increasing salinity, which was in agreement with Ranjbarfordoei *et al.* (2006). The increment of  $F_0$  and concomitant reduction of  $F_m$  indicated the impairment of light-harvesting complex in PSII, which finally reduced  $F_v$ . Reduction of  $F_v$  results in decreasing PSII quantum yield (Fernandez *et al.* 1997). With rising salinity,  $F_0/F_m$

significantly increased in both cultivars (Fig. 1), similarly to findings of Ranjbarfordoei *et al.* (2006). The ratio of  $F_0/F_m$  increases significantly in stressed and damaged plants (Bilger *et al.* 1987, Ranjbarfordoei *et al.* 2006), because of excitation energy lost during its transfer from pigment bed to the reaction centers and also due to increasing energy loss through nonphotochemical quenching processes (Yordanov *et al.* 1997, Roháček 2002). The  $F_v/F_m$  declined significantly in both cultivars with increasing stress (Fig. 2), which was in agreement with Ranjbarfordoei *et al.* (2006). Björkman and Demmig (1987) reported that  $F_v/F_m$  ratio is almost constant for many plant species under optimal conditions and it ranges between 0.80 and 0.86. Thus, our control plants were not under stress conditions. Bongi and Loreto (1989) reported that salt stress significantly reduced  $F_v/F_m$  of olive. There was a positive correlation between photosynthetic inhibition and reduction of  $F_v/F_m$  (Bongi and Loreto 1989). The significant reduction of the  $F_v/F_0$  ratio under increasing salinity reflects the effect of salt stress on efficiency of the photochemical process and electron transport chain in PSII (Yordanov *et al.* 1997, Ranjbarfordoei *et al.* 2006). The significant increment of  $F_s$  of both cultivars (data not shown) was in agreement with Ranjbarfordoei *et al.* (2006). On the other hand, reduction of the  $F_m'$  and the  $\Phi_{\text{PSII}}$  was in agreement with Jimenez *et al.* (1997), and Ranjbarfordoei *et al.* (2006). The  $\Phi_{\text{PSII}}$  indicates the photochemical capacity of PSII under light conditions. Moreover, this variable shows the actual fraction of reaction centers that are open in PSII (Krause *et al.* 1982). Juneau *et al.* (2005) found out  $\Phi_{\text{PSII}}$  proportional to the photon energy that is captured by opened reaction centers in PSII. Genty *et al.* (1989) reported  $\Phi_{\text{PSII}}$  as insensitive to some environmental factors. However,  $\Phi_{\text{PSII}}$  seemed to be the sensitive indicator of salt stress in our experiment and it was supported also by Ranjbarfordoei *et al.* (2006). Singh and Dubey (1995) stated that salt stress inhibits PSII activity. Roháček (2002) suggested that  $\Phi_{\text{PSII}}$  is related to the actual fraction of photochemically active reaction centers of PSII in light conditions. Thus, fluorescence obtained in the light-adapted state ( $F_s$  and  $F_m'$ ) is sensitive to alteration in electron transport between PSII and PSI and to biochemical reactions related to photosynthesis (Lazár 1999). In this work, the entire PSII-PSI electron transport was negatively affected in both cultivars, even more in Shishe.

Elevated  $\text{Na}^+$  concentration in current shoots of both cultivars compared to leaves might result from ion selectivity in root cell membrane and inhibition of its accumulation in leaf tissues or it is related to salt exclusion from leaves. Leaf  $\text{Cl}^-$  accumulation showed significant difference. The transport of  $\text{Cl}^-$  ion occurs mainly in the transpiration stream (Wahome 2003). As  $g_s$  of Malas was higher than that of another cultivar, the  $\text{Cl}^-$  concentration increased within the leaf. Although plants generally manage the  $\text{Na}^+$  transport better than  $\text{Cl}^-$  (Munns and Tester 2008), leaf  $\text{Cl}^-$  concentration was similar to  $\text{Na}^+$  in Malas and higher than that of  $\text{Na}^+$  in Shishe. Regarding to



Tables 5 and 6, more  $\text{Na}^+$  accumulated in current shoots than in leaves similarly to findings of Neocleous and Vasilakakis (2007) in raspberry, Goreta *et al.* (2007) in olive, Khan and Panda (2008) in rice and Sabra *et al.* (2012) in *Echinaceae*. On the other hand, chloride accumulation increased in leaves, compared to current shoots similarly to findings of Neocleous and Vasilakakis (2007) in raspberry and Sabra *et al.* (2012) in *Echinaceae*. Some reports indicated that these salts are toxic to cytoplasm at higher concentrations (Bohnert and Jensen 1996, Nuccio *et al.* 1999).  $\text{Cl}^-$  are absorbed at higher rates

than  $\text{Na}^+$  (Marschner 1995), thus  $\text{Cl}^-$  ions accumulate more than  $\text{Na}^+$  in leaf tissues (Greenway and Munns 1980). This was proved in citrus (Bar *et al.* 1998, Prior *et al.* 2007), raspberry (Neocleous and Vasilakakis 2007), soybean, avocado, and grapevine (Munns and Tester 2008).

We concluded that Malas was less affected than Shishe by osmotic effects of  $\text{NaCl}$ , as proved by growth and RWC reduction and stomata closure. However, Shishe managed  $\text{Na}^+$  and  $\text{Cl}^-$  transport into the leaves better than Malas. Thus, based on ion uptake, it seemed that Shishe might be more tolerant to salinity stress.

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